Please cite this article as: Fiona *et. al.*, (2014). Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress, *Bio-protocol* 4 (5): e1055. DOI: 10.21769/BioProtoc.1055.

Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress

Fiona Hackett, Christine R Collins, Malcolm Strath and Michael J Blackman*

Division of Parasitology, MRC National Institute for Medical Research, London, UK
*For correspondence: mblackm@nimr.mrc.ac.uk

[Abstract] Upon rupture of *Plasmodium falciparum* (*P. falciparum*) schizonts *in vitro* (an event known as egress), merozoites are released into the culture medium. The merozoites invade fresh red blood cells, a process that involves shedding of a microneme protein called apical membrane antigen-1 (AMA1) from the merozoite surface. This shedding, which takes place even in the absence of invasion, is therefore a surrogate marker for the degree of egress taking place in a culture, and can be measured using a specific capture ELISA to quantify AMA1 levels in culture supernatants (Collins *et al.*, 2013). The assay uses a monoclonal antibody specific for AMA1 (called 4G2dc1) (Kocken *et al*., 1998; Collins *et al*., 2009) to capture and immobilize the protein from culture supernatants, then uses a specific rabbit polyclonal antiserum to detect the immobilized antigen. A phosphatase-conjugated goat anti-rabbit antibody is finally used to quantify the binding of the second antibody. Egress is absolutely dependent upon the activity of a parasite cGMP-dependent protein kinase, PKG, and so is influenced by levels of intracellular cGMP (Collins *et al*., 2013). This is regulated by the interplay between guanylate cyclases and phosphodiesterases. The latter enzymes may also degrade cAMP, so it may also be informative to measure intracellular cAMP levels.

Materials and Reagents

1. *Plasmodium falciparum* schizonts
2. DetectX Direct cGMP or cAMP immunoassay kit (Arbor Assays, catalog number: K020-H1 or K019-H1)
3. Protein-free RPMI 1640 (Life Technologies, Invitrogen™)
4. Albumax
5. Zaprinast (Sigma-Aldrich, catalog number: Z0878)
   Note: Make up as a 50 mM stock solution in DMSO and stored at -20 °C.
6. PKG inhibitor compound 1 {4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1*H*-pyrrol-3-yl] pyridine} (Merck KGaA)
   Note: Make up as a 10 mM stock solution in DMSO and stored at -20 °C.
7. Anti-AMA1 monoclonal antibody (clone 4G2dc1) (Kocken *et al*., 1998)
8. Complete RPMI 1640 culture medium (Blackman, 1994)
9. Tween 20
10. Rabbit polyclonal anti-AMA1 serum (Collins *et al.*, 2009)

Please cite this article as: Fiona *et. al.*, (2014). Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress, *Bio-protocol* 4 (5): e1055. DOI: 10.21769/BioProtoc.1055.

11. Phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma-Aldrich, catalog number: A3687)
12. Phosphatase substrate (Sigma-Aldrich, catalog number: S0942)
13. Phosphate-buffered saline (PBS)
14. Sodium azide [10% (w/v) stock solution in water]
15. Dry ice-ethanol mix for freezing
16. Gelatine-tween stock solution (blocking buffer) (see Recipes)
17. 0.1 M Sodium carbonate/bicarbonate (pH 9.6) (see Recipes)
18. Diethanolamine buffer (pH 9.8) (see Recipes)

Equipment

1. Centrifuge
2. 96-well Immulon plates (Nunc®)
3. ELISA reader
4. Incubator

Procedure

A. Assay of cGMP levels in the malaria parasite
   1. For measurement of intracellular cyclic nucleotide levels, Percoll-enriched schizonts were suspended at a 5% haematocrit in gassed, protein-free RPMI 1640 at 37 °C. Standard procedures were used to culture the parasites and enrich schizonts (Blackman, 1994; Yeoh *et al.*, 2007). The suspension was divided into aliquots each containing 2 x 10^8 schizonts (2 x 10^8 schizonts corresponds to ~20 µl packed cells).
   2. Duplicate zero time samples (each containing 2 x 10^8 schizonts) were pelleted by centrifugation (1 min, 13,000 *x g*) and snap-frozen in a dry ice-ethanol mix.
   3. Further duplicate samples were then taken at various time intervals following addition of 75 µM zaprinast, zaprinast plus the 2.5 µM PKG inhibitor compound 1, or vehicle only (DMSO), and similarly processed. Zaprinast is an inhibitor of parasite phosphodiesterase (which degrades cGMP and cAMP), and so results in an increase in cGMP levels, inducing egress.
   4. All samples were stored at -70 °C until required for assay.
   5. cGMP and cAMP levels in schizont extracts were measured using a DetectX Direct cGMP or cAMP immunoassay kit, precisely as directed by the manufacturer.

B. Capture ELISA for quantitative analysis of *P. falciparum* blood-stage egress
   1. Coat 96-well Immulon plates with 100 µl/well of a stock solution of purified monoclonal antibody 4G2dc1 made up at 2.5 µg/ml in carbonate/bicarbonate buffer (pH 9.6).

Please cite this article as: Fiona *et. al.*, (2014). Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress, *Bio-protocol* 4 (5): e1055. DOI: 10.21769/BioProtoc.1055.

2. Seal the plates and incubate overnight in a humidified atmosphere at 4 °C. This and all subsequent incubations are done whilst stationary.
3. Wash the plates once with PBS 0.5% (v/v) Tween 20 (PBS/T), then block by adding 250 µl/well of a 1: 4 dilution of gelatine-Tween stock solution, and incubating for 2 h at room temperature.
4. Prepare serial 2-fold dilutions of test culture supernatant samples in a separate 96-well plate. Dilute the samples into RPMI 1640 culture medium containing 0.5% Albumax. For negative control wells, use RPMI Albumax medium only. Add samples to the washed ELISA plates, then seal and incubate the plates for 1 h at room temperature.
5. Wash plates 3x in PBS/T.
6. Make up a solution of rabbit polyclonal anti-AMA1 serum diluted 1:1,000 in PBS/T. Add 100 µl/well, then seal and incubate the plates a further 1 h at room temperature.
7. Wash plates 3x with PBS.
   Note: No Tween at this stage, as it interferes with the alkaline phosphatase activity in the next step of the assay.
8. Add 100 µl/well phosphatase-conjugated goat anti-rabbit IgG (whole molecule), diluted 1: 30,000 in PBS. Seal the plates, then incubate 1 h at room temperature.
9. Wash plates 5x with PBS (no tween) then add 50 µl/well substrate (1 tablet of phosphatase substrate, in 5 ml diethanolamine buffer). Incubate in the dark at 37 °C for at least 30 min. Determine OD_{405} using an ELISA reader.
10. Use the data to produce titration curves of antigen levels in the various culture supernatants.

Recipes

1. Gelatine-tween stock solution (blocking buffer)
   5 g Gelatine
   2.5 ml Tween-20
   150 µl 10% (w/v) sodium azide
   500 ml PBS
   Warm at 37 °C to dissolve the gelatine
   Use at 1: 4 dilution in PBS
   Stored stock at 4 °C
   May require warming before coaxing out of the bottle
2. 0.1 M Sodium carbonate/bicarbonate (pH 9.6)
   Make up the two component buffers first
   These are
   7.155 g $Na_2CO_3{\cdot}10H_2O$ made up to 250 ml water
   2.1 g $NaHCO_3$ made up to 250 ml water

Please cite this article as: Fiona *et. al.*, (2014). Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress, *Bio-protocol* 4 (5): e1055. DOI: 10.21769/BioProtoc.1055.

Mix the above buffers to obtain a buffer with a pH of 9.6

As an approximate measure, this requires ~37 ml 0.1 M $Na_2CO_3 \cdot 10H_2O$ plus 73 ml 0.1 M $NaHCO_3$

3. Diethanolamine buffer (pH 9.8)

   48.5 ml Diethanolamine

   982 µl 1 M $MgCl_2$

   2.5 ml10% (w/v) sodium azide

   Add 400 ml water then adjust pH to 9.8 with 1 M HCl before making up to a total of 500 ml with water

   Stored in the dark at 4 °C

Acknowledgments

This method is adapted from an original method described in Collins *et al.* (2013). The authors are grateful to Alan Thomas (Biomedical Primate Research Centre, Rijswijk, The Netherlands) for the gift of monoclonal antibody 4G2dc1. This work was supported by the UK Medical Research Council (U117532063), and received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement N° 242095 (EviMalAR).

References

1. Blackman, M. J. (1994). Purification of *Plasmodium falciparum* merozoites for analysis of the processing of merozoite surface protein-1. *Methods Cell Biol* 45: 213-220.
2. Collins, C. R., Withers-Martinez, C., Hackett, F. and Blackman, M. J. (2009). An inhibitory antibody blocks interactions between components of the malarial invasion machinery. *PLoS Pathog* 5(1): e1000273.
3. Collins, C. R., Hackett, F., Strath, M., Penzo, M., Withers-Martinez, C., Baker, D. A. and Blackman, M. J. (2013). Malaria parasite cGMP-dependent protein kinase regulates blood stage merozoite secretory organelle discharge and egress. *PLoS Pathog* 9(5): e1003344.
4. Kocken, C. H., van der Wel, A. M., Dubbeld, M. A., Narum, D. L., van de Rijke, F. M., van Gemert, G. J., van der Linde, X., Bannister, L. H., Janse, C., Waters, A. P. and Thomas, A. W. (1998). Precise timing of expression of a *Plasmodium falciparum*-derived transgene in *Plasmodium berghei* is a critical determinant of subsequent subcellular localization. *J Biol Chem* 273(24): 15119-15124.
5. Yeoh, S., O'Donnell, R. A., Koussis, K., Dluzewski, A. R., Ansell, K. H., Osborne, S. A., Hackett, F., Withers-Martinez, C., Mitchell, G. H., Bannister, L. H., Bryans, J. S., Kettleborough, C. A. and Blackman, M. J. (2007). Subcellular discharge of a serine

protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* 131(6): 1072-1083.